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The effect of exposure to mupirocin on the survival and recovery of sensitive *Staphylococcus aureus* strains

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SUMMARY

A significant percentage survival, representing a large number of viable cells, can occur in a Staphylococcus aureus *population exposed to concentrations of mupirocin up to 1,000 times the minimum inhibitory concentration. An elevated ratio of minimum inhibitory to minimum bactericidal concentration of mupirocin with staphylococci was recorded. Staphylococci which survive exposure to mupirocin may be less sensitive to the subsequent bactericidal action of the antibiotic. The observations may explain the re-colonisation with staphylococci seen in some studies.*

INTRODUCTION

The predominant reservoir of *Staphylococcus aureus* is man: about 15 - 35% of the population are colonised with *S aureus* in the anterior nares from which the organism can readily be disseminated to other parts of the body and to the environment. A decrease in host resistance to infection with *S aureus* can be associated with a wide variety of clinical syndromes of differing severity. The management of these infections may be complicated when multi-resistant staphylococci are encountered. Although the occurrence of multiple-antibiotic resistant strains of *S aureus* is still only about 5%, they can cause serious problems [1, 2] especially in association with hospital special care units where the opportunities for cross-infection and emergence of multiple resistance are high.

Any infection control policy aimed at reducing the incidence of staphylococcal infections due to multiple resistant strains must include attempts to eradicate nasal carriage. The topical use of antibiotics or skin disinfectants has had variable success, is unreliable, and may have inherent disadvantages in respect of resistance development.[3] Resistance to chlorhexidine, commonly used as a topical agent to reduce staphylococcal carriage, has recently been reported.[4]

A new compound, mupirocin (derived from *Pseudomonas fluorescens*, pseudomonic acid) has been shown to be effective in the eradication of *S aureus* from the anterior nares [5, 6] and the skin [7, 8] when applied as a 2% ointment. Casewell and Hill [9] have also shown that *in vitro* mupirocin behaves as a bactericidal antibiotic giving 99.9% reduction in the number of viable staphylococci within

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24 hours' exposure. The aim of this study was to investigate the effect on staphylococci of various concentrations of mupirocin at differing inoculum concentrations and at different stages of growth and to examine the characteristics of surviving bacteria.

MATERIALS AND METHODS

Mupirocin (Beecham Pharmaceuticals, Worthing) was supplied in pellet form as the sodium salt. Each pellet, weighing 20.5 mg, was dissolved in distilled water to give the appropriate antibiotic concentration, sterilised by filtration (0.22μ, Millipore UK) and used on the day of preparation.

Eighteen clinical isolates of *S aureus* were examined, in addition to two type cultures, *S aureus* (NCTC 6571) and a methicillin resistant *S aureus* (NCTC 10442). All strains were grown on nutrient agar slopes for 24 hours at 37°C, stored at 4°C and sub-cultured weekly. Mueller-Hinton (M-H) agar (CM 337), blood agar (Gibco 152-0600) containing 5% (v/v) defibrinated horse blood, and quarter-strength Ringer's solution (Oxoid BR 52) were prepared according to the manufacturer's instructions. Nutrient agar slopes (Oxoid R2) were supplied by the manufacturer.

Minimum inhibitory concentrations were carried out using the agar incorporation method. Four-hour peptone water cultures were used as inocula, and dispensed using a multipoint inoculator (Denley Instruments, Ltd) on to M-H agar plates containing two-fold serial dilutions of mupirocin. Inoculated plates were incubated aerobically at 37°C and examined for growth after 24 hours. The lowest concentration which gave a complete absence of growth was recorded as the minimum inhibitory concentration. Drug-free plates were inoculated as controls and *S aureus* NCTC 6571 and *S aureus* NCTC 10442 were included in each test set as control organisms.

Minimum bactericidal concentrations were determined using the velvet pad transfer method. The surface of each agar plate used in the previous determinations were sampled with a sterile velvet pad. The pad was then transferred on to the surface of an M-H agar plate. Following incubation at 37°C for 48 hours the minimum bactericidal concentration was calculated as the lowest concentration of antibiotic yielding no growth after velvet pad sub-culture.

Viable bacterial counts were determined by the spread plate method using quarter-strength Ringer's solution as diluent and 5% blood agar as plating medium. After incubation of plates at 37°C, results were expressed as the number of colony-forming units per ml of the bacterial suspension. To remove mupirocin from bacterial suspensions prior to enumeration of viable bacteria, unit volumes of suspension were centrifuged at 3,000 rpm for five minutes, the supernatant decanted, the cellular deposit washed once in broth, and then resuspended in broth to the original volume.

Staphylococci were grown in either M-H broth or quarter-strength Ringer's solution in the presence or absence of specified concentrations of mupirocin. Two inoculum levels were investigated, approximately 10^7 and 10^5 colony-forming units per ml. Cultures were sampled at intervals during the incubation period and viable counts performed. All experiments were carried out in triplicate.

The ability of *S aureus* to initiate growth in liquid medium after exposure to mupirocin was measured by conductance using the 112L microbiological growth analyser (Malthus Instruments, UK, Ltd). Staphylococci were exposed to

mupirocin at a concentration of 256 mg/l for different periods. The antibiotic was removed from each sample by the methods described and 1 ml of this suspension was used to inoculate 9 ml broth contained in 10 ml capacity growth cells. Identical cells containing antibiotic only or bacterial suspension only were incorporated as controls. All tests were incubated at 37°C and performed in duplicate. Changes in conductance of the growth medium due to bacterial growth were monitored and recorded; conductance measurements were made in microsiemens (μs). The interpretation of the conductance measurements in relation to microbial growth were as described in detail by Baynes, Comrie and Prain.[10] As growth proceeds, the conductivity of the medium increases, and a growth curve can be plotted of time (hours) against conductivity (μs). To test the stability of the mupirocin throughout each investigation, an aliquot of culture was taken before and after completion of each experiment. Each aliquot was filter-sterilised (0.22μ) and divided into two portions. One portion was treated with esterase in the ratio of one volume porcine liver esterase suspension (Sigma Diagnostics E-3128) to one volume of test suspension for 30 minutes in a 37°C waterbath. The other portion was untreated. Both treated and untreated samples were titrated in quarter-strength Ringer's solution (two-fold dilutions) beyond the minimum inhibitory concentration of the organism used. Single drops of 25 μl of each titration dilution were dispensed on to the surface of pre-dried blood agar plates and allowed to absorb into the medium undisturbed. After absorption, the plates were flooded with a log phase broth culture of *S aureus* NCTC 6571 incubated 18 hours at 37°C. Plates were examined for zones of clearing indicating the presence of the antibiotic.

RESULTS

The survival of *S aureus* NCTC 6571 in M-H broth when exposed to concentrations of 2, 16 and 256 mg/l mupirocin is shown in Fig 1. Using both high and low inoculum levels it was shown that after an initial increase in viable count over a three-hour period, a stationary phase of growth was established for a period of 24 hours. Where the initial inoculum level was exposed to 16 or 256 mg/l mupirocin, the viable count fell to less than 10 colony-forming units per ml, representing at least a 99.9% reduction of viable cells. For cells exposed to 2 mg/l mupirocin a 99.8% reduction in viable count was achieved. Where the higher inoculum level was examined, there was a reduction in viable count of 0, 98 and 99.6% after exposure to concentration of 2, 16 and 256 mg/l mupirocin. Under the conditions described with the higher inoculum level, reductions in viable count of 98% and 99.6% corresponded to counts of surviving bacteria after 120 hours' exposure to mupirocin of 2×10^5 and 4×10^4 colony-forming units per ml respectively.

The survival kinetics for a further four strains of *S aureus* showed a similar pattern to that obtained for *S aureus* NCTC 6571. The influence of these inoculum sizes on the percentage survival of three multiple-resistant (including methicillin resistance) staphylococci and *S aureus* NCTC 10442 was studied after 64 hours' continuous exposure to 256 mg/l mupirocin, and significant numbers of all strains survived this exposure. A higher percentage survival was found with the higher inoculum level. No significant difference in results was obtained with inocula prepared from lag phase, logarithmic phase or stationary phase cells. The effect of mupirocin (256 mg/l) on the survival of staphylococci at different inoculum levels in M-H broth and in quarter-strength Ringer's solution is shown

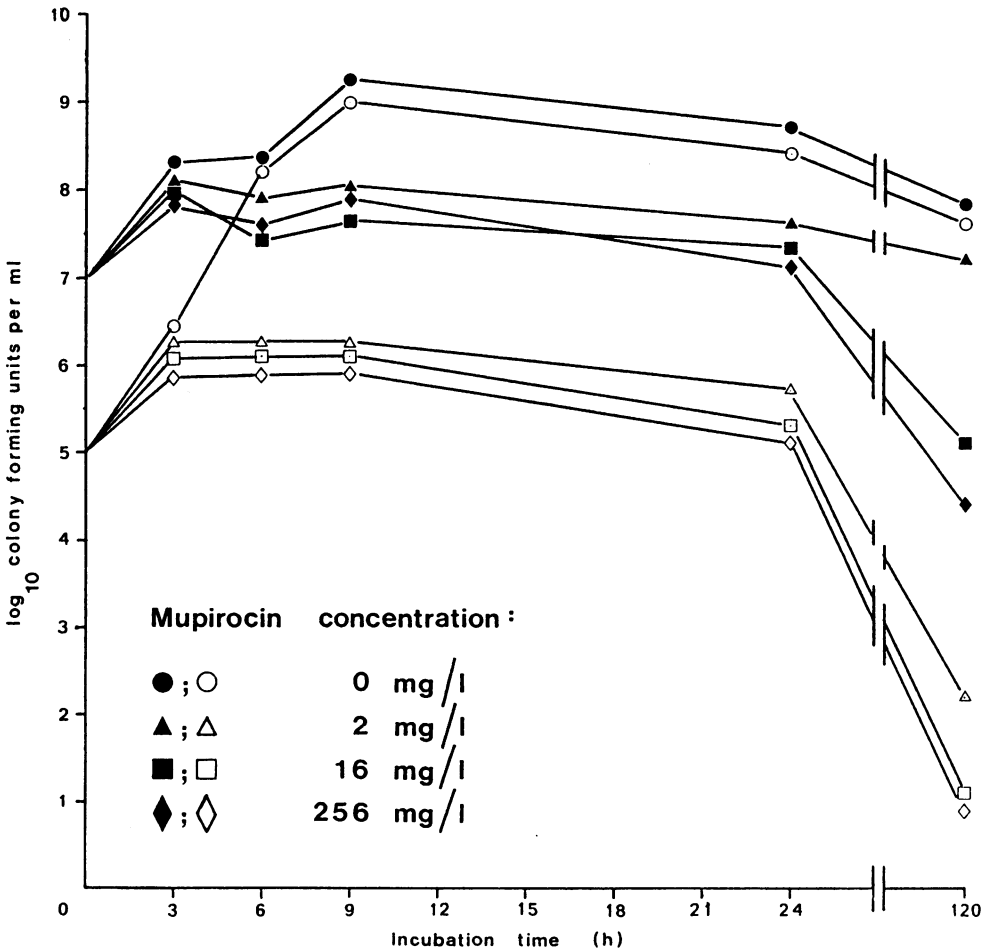

Fig 1. The effect of mupirocin on the survival of *S aureus* NCTC 6571 at two inoculum levels (1×10^7 colony-forming units per ml, black symbols; 1×10^5 colony-forming units per ml, open symbols).

in Fig 2. Cells grown in M-H broth alone (Fig 2B) demonstrated a normal growth curve for uninhibited cells, whereas cells exposed to 256 mg/l mupirocin in M-H broth showed a stationary phase of growth established over 24 hours followed by a decline in viable count. The decline in viable count shown in M-H broth confirmed the effect of inoculum on the percentage survival after 24 hours' and 120 hours' exposure to mupirocin. The viability of cells in quarter-strength Ringer's solution began to decline, even in the absence of mupirocin (Fig 2A). This was most marked with the lower initial inoculum level. However, in the presence of mupirocin, no surviving cells could be detected after 48 hours with the lower initial inoculum level or after 120 hours with the higher initial inoculum level.

A relationship was established between the inoculum size of *S aureus* NCTC 6571 and time of detection of conductance change using the Malthus microbiological growth analyser. The detection time varied between three hours and 15 hours for

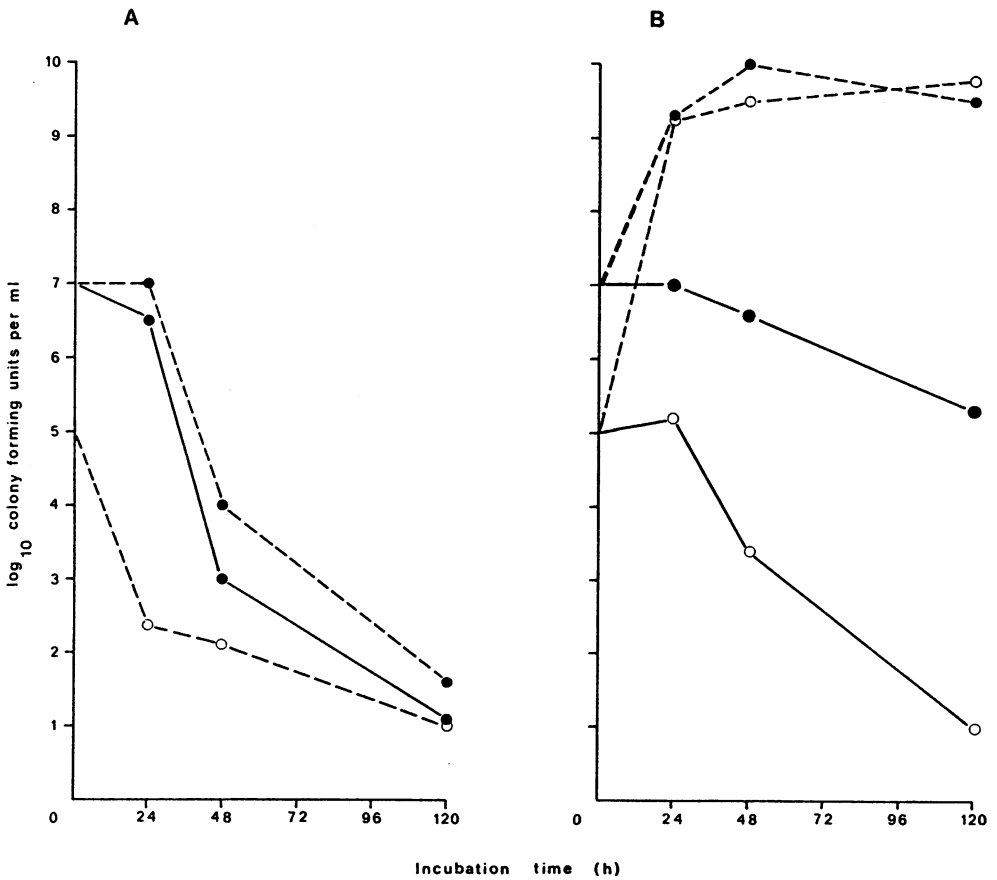

Fig 2. Survival of *S aureus* NCTC 6571 at two different inoculum levels with and without concomitant exposure to mupirocin at a concentration of 256mg/l. Both a non-nutritional (quarter-strength Ringer's solution — Fig 2A) and nutritional (M-H broth — Fig 2B) environment were tested. The initial inoculum contained cells in a logarithmic phase of growth.

Inoculum levels: 1×10^7 colony-forming units per ml (black symbols), 1×10^5 colony-forming units per ml (open symbols). Solid line ——— with mupirocin, dotted line — — — without mupirocin.

inocula varying from 2×10^9 to 2×10^2 colony-forming units per ml. *S aureus* cells exposed to mupirocin (2mg/l, 16mg/l, or 256mg/l) for different periods (24, 48 and 72 hours) showed no residual effect of mupirocin. In each case the time of detection of conductance change was as expected from the calculated viable count of mupirocin-exposed cells, and the pattern and magnitude of conductance of cells pre-exposed to mupirocin was the same as the control unexposed organism.

The minimum inhibitory and minimal bactericidal concentrations of mupirocin were determined before and after treatment of *S aureus* cultures for 24 hours with the same drug (Table). When the pre-treated cells were recovered on antibiotic-free M-H agar after overnight incubation at 37°C, no change in their susceptibility to mupirocin was noted. The minimal bactericidal concentration was generally four-fold higher than the minimal inhibitory concentration. When

TABLE

The effect of previous exposure to mupirocin on the minimum inhibitory concentrations (MIC) and minimal bactericidal concentration (MBC) of mupirocin on Staphylococcus aureus *(18 clinical isolates, and two control cultures, NCTC 6571 and 10442)*

	*Study A**		*Study B*		*Study C*	
	Previous exposure		*Previous exposure with cells washed*		*No previous exposure*	
	MIC	MBC	MIC	MBC	MIC	MBC
18 clinical isolates (mean and range)	0.5 (0.25-64)	2.0 (2-64)	0.13 (0.03-32)	64 (16-64)	0.5 (0.25-64)	2.0 (2-64)
2 control cultures (mean)	0.4	2.0	0.03	64	0.4	2

*The previously exposed staphylococci had been grown in a medium containing 256 mg/l mupirocin for 24 hours; the cells were then grown on M-H agar for 16 hours without the antibiotic before further inoculation (Study A), or simply washed free of the antibiotic (Study B). The cells in Study C had not previously been exposed to mupirocin.

mupirocin-treated cells were washed to remove the antibiotic and used directly as an inoculum, the minimal inhibitory concentration generally decreased 1–2 fold; the bactericidal concentration however, exceeded the inhibitory concentration by a factor of 500 for all clinical isolates and type cultures.

No variation in assays of mupirocin activity before and after each experiment was found. This confirmed the stability of the antibiotic in the presence of both quarter-strength Ringer's solution and M-H broth and at 37°C incubation. Bacterial degradation of the antibiotic was not apparent even when broth cultures were tested after several days' continuous incubation of 37°C. Treatment of culture filtrates with esterase negated the titration value in all cases, indicating the continued presence of the antibiotic.

DISCUSSION

S aureus is one of the major causes (20%) of hospital infection[11] which can become life-threatening in debilitated patients.[12] The re-emergence of multiple-resistant, including methicillin-resistant, staphylococci has placed further burdens both on the patient and on hospital personnel with its associated problems in the control of cross-infection.[11, 13] Such were these problems that recently detailed guidelines for the prompt control and management of methicillin-resistant *S aureus* infections were published by the Hospital Infection Society and British Society for Antimicrobial Chemotherapy (1986).[14]

Although staphylococci can be found associated with a wide range of hospital-acquired infection from skin sepsis to endocarditis, the main reservoir for these organisms is the anterior nares, and recently the importance of gastro-intestinal carriage in hospitalised patients has been reported.[15] The problem of carriage of *S aureus* has been reflected in the varied treatment regimens and measures used to control or eliminate this carriage, often resulting in organisms becoming antibiotic-resistant.[3]

The new topical antibiotic mupirocin seems to satisfy a number of criteria expected of a topical agent. It has a novel mode of action, selective anti-bacterial activity, a total lack of cross-resistance to other antibiotics, and is metabolised and excreted quickly as a non-toxic compound. Another favourable property is its action against multiple-resistant coagulase-positive and coagulase-negative staphylococci.[9] White et al (1984) stated that using experimental inocula of 10^5 colony-forming units per ml, more than 99.9% of *S aureus* were killed in 24 hours.[16] For a number of strains, using similar inoculum concentrations, Casewell and Hill (1984) agreed with this observation, demonstrating that at concentrations of 16mg/l a 99.9% reduction in viable count was achieved by 24 hours and sterility of the culture by 120 hours.[9] In this study we found that with inoculum levels of 10^5 to 10^7 colony-forming units per ml, a significant percentage survival, representing a large number of viable cells, could occur in an *S aureus* population exposed to concentrations of mupirocin up to 256mg/l (equivalent to 1,000 times the minimum inhibitory concentrations). This was demonstrated with a number of strains, including methicillin-resistant *S aureus.*

Surviving cells, if transferred to an environment free of mupirocin, showed the same growth characteristics as unexposed cells. With high inoculum levels, even exposure to 256mg/l for 72 hours could be followed by a rapid recovery when removed to an antibiotic-free environment.

A significant difference was noted between the effect of mupirocin in *S aureus* grown in broth and in quarter-strength Ringer's solution. In quarter-strength Ringer's solution, the slope of the curve for both exposed and non-exposed cells were very similar, suggesting a direct effect of the Ringer's environment. However, no viable cells were recovered after 120 hours, when the initial population of cells were exposed to mupirocin (256mg/l) in a Ringer's salts environment; this suggested that the action of the antibiotic was not altogether dependent on the presence of bacteria in a phase of rapid growth and protein synthesis. This was confirmed by the findings that the antibiotic effect on viable counts of bacteria was not significantly different using lag, logarithmic or stationary phase cells. The ratio of minimal inhibitory to minimal bactericidal concentration of mupirocin with staphylococci is quoted at between 1:8 and 1:32. Our findings demonstrate a ratio of at least 1:128 for cells that have been treated with mupirocin and used directly as inoculum. It appears that cell growth in the presence of mupirocin ceases and will only resume on removal of the antibiotic. The change in the ratio in this case seems to confirm that cells surviving exposure to mupirocin at high concentrations are less sensitive to the subsequent bactericidal action of mupirocin. It is possible that in an *in vitro* situation the cells which survived an initial challenge with high concentrations of mupirocin could be less sensitive to the bactericidal action of subsequent exposure.

These results do not differ very much from the observations of other workers, although with similar inocula we cannot demonstrate such a high percentage loss of viability in the presence of mupirocin. This may be due to some carry-over of mupirocin on plating media. Cells are sufficiently damaged to be inhibited from rapid growth on solid media, but viability can often be demonstrated by velvet pad transfer to fresh media or by prior centrifugation of treated organisms to remove the antibiotic. At inoculum levels higher than 10^5 we find that, even at high mupirocin concentrations, high numbers of organisms can survive for 120 hours and retain their ability to grow again in a manner similar to untreated cells, when environmental conditions become favourable.

Although the antibacterial properties of mupirocin support its use as a topical antibiotic, we feel that, in some clinical applications (where high concentrations of mupirocin locally challenge high numbers of staphylococci at a micro-environment level), sufficient numbers of viable organisms may remain to initiate re-colonisation. This may explain the low percentage re-colonisation with the same strain of staphylococcus seen in some studies.

The authors would like to thank Beecham Laboratories for supplies of mupirocin ointment, Professor A M Emmerson for his assistance and Mrs C McIlhatton for typing the manuscript. Mupirocin is marketed as Bactroban (Beecham), which is a 2% ointment in a water-miscible macrogel base.

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